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Pantothenate auxotrophy of *Methylobacterium* spp. isolated from living plants

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Abstract

A number of pink-pigmented facultative methylotrophs (PPFMs) belonging to *Methylobacterium* spp. isolated from living plant samples were found to require B vitamins for their growth in minimal medium, and most B vitamin-auxotrophic PPFMs required pantothenate (vitamin B_5). Further investigation of pantothenate auxotrophy using the representative strain *Methylobacterium* sp. OR01 demonstrated that this strain cannot synthesize β-alanine, one of the precursors of pantothenate. β-alanine and several precursors of pantothenate restored the growth of *Methylobacterium* sp. OR01 in minimal medium. Furthermore, this strain could colonize leaves of *Arabidopsis thaliana* cultivated in medium without pantothenate or its precursors. Pantothenate, β-alanine and several precursors were detected in the suspension of *A. thaliana* leaves. These results suggest that pantothenate-auxotrophic PPFMs can symbiotically colonize the surface of plant leaves by acquiring β-alanine and other precursors, in addition to pantothenate. Finally, the fitness advantage of B vitamin auxotrophy of PPFMs in the phyllosphere environment is discussed.

Key words: B vitamin auxotrophy, pantothenate, β-alanine, *Methylobacterium*, phyllosphere

Introduction

The phyllosphere, which comprises the above ground part of terrestrial plants, provides an extensive habitat for microorganisms. In the phyllosphere, plants are expected to supply all of the nutrients and cofactors required for the growth and survival of phyllospheric microorganisms. Plants are known to produce various kinds of volatile organic compounds (VOCs) [1, 2]. Among them, methanol is abundantly produced from the methyl ester groups of the plant cell wall component pectin. Methanol present on plant leaves can be utilized as carbon sources by a subset of phyllospheric microorganisms, called methylotrophs [3]. Our previous study using a methylotrophic yeast methanol sensor revealed that the methanol concentration present on the leaf surfaces of *Arabidopsis thaliana* is oscillated in the range of 0-0.2% (ca., 0-60 mM) [4]. The presence of sugar compounds including glucose on the leaf surface has also been reported [5]. In addition to these compounds, nitrate, methylamine, and amino acids, which can be used as nitrogen sources, and some trace cofactors like vitamins, have also been reported to be present on the leaf surface [6, 7].

The leaf surface is the main area of the phyllosphere and it harbors a unique microbial community. The bacterial population on the leaf surface is estimated to be 10^6 - 10^7 cells/cm^2, while archaea and fungi also colonize on leaves at smaller population sizes than bacteria [8]. Delmotte *et al*. reported that the dominant class of phyllosphere bacteria on soybean leaves was Alphaproteobacteria, which represented more than 40% of the population, and included *Sphingomonas* and *Methylobacterium* as the major species [9]. *Methylobacterium* spp. can utilize methanol as the sole carbon and energy source and are also called pink-pigmented facultative methylotrophs (PPFMs). These bacteria are known to have the ability to promote plant growth through the production of phytohormones, such as auxins and cytokinins, and induce systemic resistance against pathogens and diseases [10-14]. Thus, much attention has

recently been paid to the symbiotic interaction between *Methylobacterium* spp. and plants, and their potential applications.

The ability of *Methylobacterium* spp. to grow on methanol is thought to be one of the reasons why these bacteria can dominate on leaf surfaces. In previous studies, the relationship between the ability to grow on methanol and the ability to colonize plant leaf surfaces was investigated using the representative model strain *Methylobacterium extorquens* AM1, which was originally isolated as an airborne contaminant growing on methylamine [15] and has recently been re-classified as *Methylorubrum extorquens* [16]. Competition experiments with wild-type and mutant strains of *M. extorquens* AM1 revealed that mutant strains lacking *mxaF* or *xoxF*, encoding the large subunit of the Ca^{2+}-dependent methanol dehydrogenase (MDH) or a lanthanide-dependent MDH, respectively, are less competitive than the wild-type strain during colonization on plant leaves [17, 18]. These findings suggest that the ability to utilize methanol as a carbon source is advantageous for *Methylobacterium* spp. for proliferation and survival in the phyllosphere.

So far, a number of PPFMs have been isolated from various living plant samples [19-23]. We have investigated the distribution of PPFMs on the leaves and seeds of various vegetables, and found that red perilla plants harbor a dominant population of PPFMs, with *Methylobacterium* sp. OR01 as a representative strain [24]. We also confirmed direct transmission of *Methylobacterium* sp. OR01 from red perilla seeds to their leaves, and this strain exhibited a greater ability to colonize on the red perilla plant than *M. extorquens* AM1 [25]. In general, media for screening or enrichment culture of PPFMs contain a mixture of B vitamins [26-28]. However, our knowledge of the auxotrophic requirements for vitamins and other cofactors by various *Methylobacterium* spp. is limited. It was reported that the addition of pantothenate (vitamin B_5) enhanced the growth of several strains of *Methylobacterium* spp. [29], but there have been no reports whether pantothenate auxotrophy is relevant to the growth environment in the phyllosphere.

In this study, we found that many PPFMs isolated from living plant samples exhibit B-vitamin auxotrophy, especially pantothenate auxotrophy, and the representative *Methylobacterium* sp. OR01 could grow on the leaf surface of the model plant *Arabidopsis thaliana*. The availability of biosynthetic precursors of pantothenate was predicted from the draft genome sequence of *Methylobacterium* sp. OR01, and we demonstrated that the pantothenate auxotrophic *Methylobacterium* sp. OR01 can utilize pantothenate or its precursors present on the surface of *Arabidopsis* leaves. Finally, a fitness advantage of the pantothenate auxotrophic PPFMs compared to the non-auxotrophic *M. extorquens* AM1 in the phyllosphere environment was identified and discussed.

Materials and Methods

Bacterial strains and culture conditions. *Methylobacterium* sp. OR01 [25], *M. extorquens* AM1 [15], and other *Methylobacterium* spp. strains isolated from plant samples (Table 1; details of the screening procedure and characterization of each strain will be described elsewhere) were grown at 28°C in Hypho minimal medium (MM) [30] supplemented with 0.5 % (v/v) methanol or 0.2% (w/v) disodium succinate as the carbon source. Single B-group vitamins or B-vitamin mixtures (VB mix) were added to MM. The B-group vitamins were thiamine (B_1), niacin (B_3), Ca-pantothenate (B_5), pyridoxine HCl (B_6), biotin (B_7), cobalamin (B_{12}), inositol (B-h), and *p*-amino benzoate (B-x); concentrations in the medium were 4 μg/mL, 4 μg/mL, 4 μg/mL, 2 μg/mL, 20 ng/mL, 4 μg/mL, 2 μg/mL, and 2 μg/mL, respectively.

Measurement of populations of PPFMs on Arabidopsis thaliana *leaves.* *Methylobacterium* sp. OR01 and *M. extorquens* AM1 were grown in MM containing 0.5% methanol and VB mix at 28°C for 3 days. The bacterial cultures were collected and washed twice with sterilized water and then resuspended in sterilized water to obtain cell suspension with an OD_{600} of 0.1.

Arabidopsis seeds were sterilized with 70% ethanol for 30 sec followed by 0.5% hypochlorite solution for 15 min. They were mixed with the cell suspension and gently shaken by using a rotating mixer (Rotator RT-5, Taitec, Saitama, Japan) for 2 h. Seeds inoculated with *Methylobacterium* sp. OR01 or *M. extorquens* AM1 were placed on agar plate medium, Murashige and Skoog medium (Duchefa, Haarlem, Netherlands) supplied with 0.8 % Bacto agar (Difco Becton Dickinson, Franklin Lakes, NJ, USA), which did not contain pantothenate or its precursors. The seeds were aseptically incubated in a plant growth chamber (Nippon Medical & Chemical Instruments, Osaka, Japan) at 25°C for 14 days in a daily light/dark cycle (14 h light/10 h dark). The

fourth leaves were removed from the plants and soaked in 500 µL of sterilized water. Bacterial cells colonizing on the leaf surface were removed from the leaves by vortexing for 2 min and incubating in an ultrasonic bath (UT205S, Sharp, Osaka, Japan) for 15 min. The supernatant was serially diluted and plated onto MM agar medium containing 0.5% methanol and VB mix. Plates were incubated at 28°C for 4-5 days and then the number of pink colonies was counted and the colony forming units (CFU/leaf) were determined.

Quantitative analysis of compounds on the leaf surface. After *A. thaliana* was grown on Murashige and Skoog agar medium lacking pantothenate in a plant growth chamber at 25°C for 14 days, ten leaves were separated from plants and the cut edges were sealed with instant glue (Aron Alpha EXTRA, Toagosei, Tokyo, Japan) to prevent compounds from flowing out of leaves. These leaves were soaked in 500 µL of sterilized water for 10 min to collect compounds present on the leaf surface. The amount of pantothenate, spermine, spermidine, 5,6-dihydrouracil, *N*-carbamoyl-β-alanine, and 3-hydroxypropanoate in the collected solution was measured by LC-MS/MS analysis. The collected solution was subjected to chromatographic separation on a Hydrosphere C18 column (column size 2 × 150 mm, YMC, Kyoto, Japan) with a Prominence UFLC HPLC system (Shimazu, Kyoto, Japan). The mobile phase used was H_2O containing 0.05% acetic acid (solvent A) and methanol (solvent B). The gradient conditions for B% were: 0–6.0 min = 2%, 6.0–10.0 min = 2–80%, 10.0–12.0 min = 80–2%, 12.0–20.0 min = 2% for *N*-carbamoyl-β-alanine, 3-hydroxypropanoate, and pantothenate analysis, and 0–10.0 min = 2% for spermine, spermidine, and 5,6-dihydrouracil analysis. The flow rate was 0.1 mL/min and the injection volume was 5 µL.

β-alanine was derivatized with 3-aminopyridyl-*N*-hydroxysuccinimidyl carbamate (APDS) regent (Amino Acid Analysis Reagent, FUJIFILM Wako Pure

Chemical, Osaka, Japan) and subjected to chromatographic separation on a Wakopak® Wakosil-II 3C8-100HG column (column size 2 x 100 mm, FUJIFILM Wako Pure Chemical) [31]. The mobile phase used was APDSTAG Wako Eluent (solvent A, FUJIFILM Wako Pure Chemical) and 60% acetonitrile solution (solvent B). The gradient conditions for B% were: 0–1.25 min = 4%, 1.25–1.26 min = 4-15%, 1.26-5.0 min = 15-20%, 5.0-5.5 min = 20-50%, 5.5-6.5 min = 50-95%, 6.5-6.75 min = 95%, 6.75-6.76 min = 95-4%, 6.76-12.0 min = 4%. The flow rate was 0.3 mL/min and the injection volume was 2 μL.

Detection was conducted with a 4000 QTrap with turboionspray source (AB Sciex, Framingham, MA, USA). Data acquisition for all experiments was carried out by Multiple Reaction Monitoring (MRM) method operated with Analyst software ver. 1.5 (AB Sciex). The setting parameters were shown in Table S1.

Results

Pantothenate auxotrophy of PPFMs isolated from living plant samples

Of the 13 tested strains that were isolated from living plant samples, 10 strains (By, B2B, RS1, C06, C16, C17, In1, OR01, Sh, and Rst) required the B-group vitamins mixture (VB mix) for their growth on Hypho minimal medium (MM), and the other 3 strains (C1, Dw, and Rfw) did not (Table 1). Next we investigated which B-vitamins could recover the growth of each strain on MM. Results showed that the addition of pantothenate (vitamin B_5) restored the growth of 9 strains on MM (Table 1). The 16S rRNA gene sequence analysis revealed that these 9 strains belong to *M. radiotolerans* and *M. fusisawaense* (isolation and identification of these strains will be described elsewhere). *Methylobacterium* sp. Rst required both biotin (vitamin B_7) and pyridoxine (vitamin B_6) for its growth on MM. These results indicate that many *Methylobacterium* spp. that exhibit B-group vitamins auxotrophy, could inhabit the phyllosphere.

Biosynthetic pathway for pantothenate in Methylobacterium *sp. OR01*

Because the pantothenate auxotrophs were closely related to each other, further investigation of pantothenate auxotrophy was conducted using the representative strain *Methylobacterium* sp. OR01, which was isolated from red perilla seeds [25] and whose draft genome sequence was determined (details will be described elsewhere). Pantothenate is synthesized by a condensation reaction of pantoate and β-alanine in bacteria, by pantothenate synthetase (EC: 6.3.2.1) encoded by the *panC* gene (Figure 1(a)) [32]. First, we examined whether *Methylobacterium* sp. OR01 required pantoate or β-alanine for its growth in minimal medium. *Methylobacterium* sp. OR01 was able to grow in MM supplemented with β-alanine, although the maximum OD_{600} of this strain when supplemented with β-alanine was lower than that with pantothenate. In

contrast, *Methylobacterium* sp. OR01 did not grow with pantoate (Figure 1(b)). These results indicate that the pantothenate auxotrophy of *Methylobacterium* sp. OR01 is due to the inability to synthesize β-alanine.

Several biosynthetic pathways for β-alanine are known in bacteria, in which β-alanine is synthesized from L-aspartate, putrescine, uracil, α-alanine, malonate, oxalate, and lactate (Figure 2) [33, 34]. To identify the defective portion of the β-alanine biosynthetic pathways in *Methylobacterium* sp. OR01, genes encoding putative enzymes involved in β-alanine biosynthesis were searched in the draft genome sequence. In the pathways from uracil and putrescine, homologs of β-alanine biosynthetic enzyme genes were found in *Methylobacterium* sp. OR01 (red arrows in Figure 2). It is noteworthy that *Methylobacterium* sp. OR01 has homologs of genes for all enzymes in the β-alanine biosynthetic pathway from uracil via 3-oxopropanoic acid, although this strain cannot synthesize β-alanine. Amino acid sequences of the genes annotated as enzymes involved in the pathway from uracil to β-alanine in *Methylobacterium* sp. OR01 exhibited more than 60% similarity with those characterized in *E. coli* or *Pseudomonas aeruginosa* (data not shown). It is possible that enzymes in *Methylobacterium* sp. OR01 show different substrate specificity or these genes are not expressed in this strain.

Next, to identify precursors of β-alanine that recover the growth of *Methylobacterium* sp. OR01 in minimal medium, the strain was cultured in MM supplemented with 12 different compounds, lactate, L-aspartate, spermine, spermidine, putrescine, uracil, 5,6-dihydrouracil, *N*-carbamol-β-alanine, α-alanine, malonate, oxalate, and 3-hydroxypropanoate (Figure 2). Among them, addition of spermine, spermidine, 5,6-dihydrouracil, *N*-carbamol-β-alanine, and 3-hydroxypropanoate could recover the growth of *Methylobacterium* sp. OR01 in MM (Figure 3). The growth yields of *Methylobacterium* sp. OR01 supplemented with spermine or spermidine were lower than that with 5,6-dihydrouracil, *N*-carbamol-β-alanine, or 3-hydroxypropanoate.

On the other hand, all starting compounds for β-alanine biosynthesis, including L-aspartate, putrescine, uracil, α-alanine, malonate, oxlate, and lactate, could not recover the growth of *Methylobacterium* sp. OR01 on MM. Judging from these results, *Methylobacterium* sp. OR01 can utilize β-alanine in addition to spermine, spermidine, 5,6-dihydrouracil, N-carbamoyl-β-alanine, or 3-hydroxypropanoate, as the precursors of pantothenate biosynthesis.

Colonization of pantothenate auxotrophic Methylobacterium *sp. OR01 on the surface of* Arabidopsis *leaves*

In our previous study, we revealed that *Methylobacterium* sp. OR01 is a dominant colonizer on the red perilla plant [24]. Our present results demonstrating that *Methylobacterium* sp. OR01 could utilize β-alanine and its precursors suggest that this strain acquires pantothenate or its precursors from the plant surface environment. To confirm that *Methylobacterium* sp. OR01 acquires pantothenate or its precursors from plants, we inoculated cells of *Methylobacterium* sp. OR01 on sterilized seeds of the model plant *A. thaliana* and cultivated the plant on medium lacking pantothenate and its precursors. After two-weeks of cultivation, the leaves were suspended in sterilized water and sonicated to release bacterial cells from the plant surface, and the colony forming units (CFU/leaf) of the cell suspension were determined as described in Materials and Methods. *M. extorquens* AM1, a pantothenate prototroph and model *Methylobacterium* sp. strain was used for comparison (Table 1). The suspension from leaves inoculated with *Methylobacterium* sp. OR01 contained 7.9×10^4 (2.8×10^4 - 1.8×10^5) CFU/leaf, which was comparable to that with *M. extorquens* AM1, 2.7×10^4 (1.0×10^4 - 7.8×10^4) CFU/leaf (Figure 4(a)). These results indicate that the pantothenate-auxotrophic *Methylobacterium* sp. OR01 acquired sufficient pantothenate or its precursor compounds to allow proliferation during colonization on *Arabidopsis* plant. In addition, the dominant colonization of *Methylobacterium* sp. OR01 over *M.*

extorquens AM1 on the *Arabidopsis* leaves was observed as shown in Figure 4(b) (see Discussion).

Pantothenate and its precursors are present on the surface of Arabidopsis *leaves*

To investigate whether a sufficient amount of pantothenate and/or its precursors to compensate for the pantothenate auxotrophy of *Methylobacterium* sp. OR01 is present on the plant leaf surface, we used LC-MS/MS analysis to quantify the amounts of pantothenate, β-alanine and its precursors, spermine, spermidine, 5,6-dihydrouracil, *N*-carbamoyl-β-alanine, and 3-hydroxypropanoate, which were washed off of the surface of *Arabidopsis* leaves into the leaf-suspension solution. The presence of significant amounts of pantothenate, β-alanine, spermidine, *N*-carbamoyl-β-alanine, and 3-hydroxypropanoate were detected, while spermine and 5,6-dihydrouracil were below the limit of detection (Table 2). The amount of β-alanine (1.1×10^3 pmol/g fresh leaves) was c.a. 100-fold greater than that of pantothenate (1.5×10^1 pmol/g fresh leaves) and much higher than that of β-alanine precursors. Judging from these results, the pantothenate auxotrophic *Methylobacterium* sp. OR01 appears to acquire pantothenate from the plant and also acquires β-alanine for use in pantothenate biosynthesis.

Ability of Methylobacterium *sp. OR01 to utilize pantotheanate and β-alanine*

To predict which compounds, pantothenate or β-alanine, contribute to the growth of *Methylobacterium* sp. OR01 in the phyllosphere, we investigated the ability of this strain to utilize pantothanate and β-alanine. *Methylobacterium* sp. OR01 was cultivated in MM supplemented with various concentrations of pantothanate or β-alanine. *Methylobacterium* sp. OR01 could grow in the presence of more than 1 μM pantothenate or more than 5 μM β-alanine (Figure 5). The maximum growth rate was observed when the medium was supplemented with 5 μM pantothenate or 45 μM

278 β-alanine. Thus, the concentration of pantothenate that supported the optimal growth
279 of *Methylobacterium* sp. OR01 was c.a. 10-fold lower than that of β-alanine. However,
280 since the concentration of β-alanine on the leaf surface was 100-fold higher than that of
281 pantothenate, *Methylobacterium* sp. OR01 is thought to utilize both pantothenate and
282 β-alanine in the phyllosphere.
283

Discussion

Phyllosphere microorganisms utilize various nutrient compounds present on plant leaves for their growth and survival in the phyllosphere. For example, methanol and sugar compounds, including glucose, can be utilized as carbon sources, and nitrate and methylamine can be utilized as nitrogen sources [3, 5, 6, 35]. However, it was unknown how phyllosphere microorganisms utilize low molecular compounds that function as cofactors or vitamins in the phyllosphere. In this study, we focused on B vitamin auxotrophy of *Methylobacterium* spp., which are known to be the dominant colonizers in the phyllosphere, and revealed that a number of *Methylobacterium* strains isolated from living plant samples required B vitamins, especially pantothenate (vitamin B_5), for their growth.

According to the KEGG pathway database (https://www.genome.jp/kegg/pathway.html), all enzymes involved in pantothenate biosynthesis except ketopantoate reductase, which generates pantoate from ketopantoate, are encoded in the genome of *Arabidopsis*, soybean, rice, tomato, and potato [36]. Generation of pantoate from ketopantoate is thought to be catalyzed not by ketopantoate reductase, but by acetohydroxy acid isomeroreductase encoded by the *ilvC* gene in *A. thaliana* [37]. Therefore, plants are assumed to be able to synthesize pantothenate. It was also reported that not only pantothenate but also other B vitamins, including thiamine and pyridoxine, are present on leaves of *Sambucus nigra* [7]. Therefore, B vitamin auxotrophs living in the phyllosphere are assumed to utilize B vitamins synthesized by plants. Indeed, pantothenate auxotrophic *Methylobacterium* sp. OR01 was shown to utilize pantothenate or its precursors from the leaf surface environment of *Arabidopsis* (Figure 4(a) and Table 2).

The growth of the pantothenate auxotrophic *Methylobacterium* sp. OR01 on minimal medium was recovered by the addition of β-alanine, one of the precursors of

311 pantothenate biosynthesis (Figure 1(b)). Several putative genes encoding enzymes
312 involved in β-alanine biosynthesis were found in the draft genome sequence of
313 *Methylobacterium* sp. OR01 (Figure 2), and spermine, spermidine, 5,6-dihydrouracil,
314 *N*-carbamoyl-β-alanine, and 3-hydroxypropanoate were found to restore the ability of
315 *Methylobacterium* sp. OR01 to grow in minimal medium (Figure 3). These results
316 suggest that multiple pathways can function in β-alanine biosynthesis in
317 *Methylobacterium* sp. OR01. The ability of the pantothenate auxotrophic strain to
318 synthesize β-alanine not from a single compound but from several different compounds
319 may enable this strain to adapt to various environments including the phyllosphere.
320 We demonstrated that the pantothenate auxotrophic *Methylobacterium* sp. OR01
321 could colonize *Arabidopsis* leaves cultivated on plant medium which did not contain
322 pantothenate or its precursors (Figure 4(a)) and that pantothenate, β-alanine, and several
323 precursor compounds were present on *Arabidopsis* leaves (Table 2). These results
324 suggest that *Methylobacterium* sp. OR01 can acquire pantothenate and/or its precursors
325 produced by host plants. We also revealed that the amount of β-alanine on
326 *Arabidopsis* leaves was ca. 100-fold higher than that of pantothenate and other
327 precursors (Table 2) and that the minimum concentrations of pantothenate and β-alanine
328 necessary to support the growth of *Methylobacterium* sp. OR01 in minimal medium
329 were almost the same (Figure 5). The concentrations of pantothenate and β-alanine on
330 the leaf surface cannot be estimated by the values indicated in Table 2, however,
331 enough amount to support the growth of bacterial cells is thought to be constantly
332 supplied in the phyllosphere. Therefore, not only pantothenate but also β-alanine
333 produced by the host plant, could support the proliferation of the pantothenate
334 auxotrophic *Methylobacterium* sp. OR01 on plant surfaces.
335 Regarding the uptake of pantothenate and β-alanine by *Methylobacterium* sp.
336 OR01, homologs of PanT and PanF, which are bacterial pantothenate transporters [38,
337 39], were not found in the draft genome sequence of *Methylobacterium* sp. OR01.

However, a gene encoding a putative amino acid permease, which has 43% amino acid sequence similarity to the β-alanine transporter CycA of *E. coli* [40], was found, and might be involved in uptake of pantothenate.

In our previous study, the red perilla colonization ability of *Methylobacterium* sp. OR01, which was originally isolated from red perilla seeds, was compared with that of *M. extorquens* AM1 [25]. Sterilized red perilla seeds were inoculated with *Methylobacterium* sp. OR01, *M. extorquens* AM1, or a mixture of both strains and the CFUs in the suspension of leaves were determined after two-weeks of cultivation of the red perilla plant. Although both strains colonized red perilla leaves when inoculated separately onto the seeds, only *Methylobacterium* sp. OR01 colonized leaves when the mixture was inoculated [25]. In this study, we conducted the same experiments using the model plant *A. thaliana,* and the presence of *Methylobacterium* sp. OR01 alone on leaves was confirmed in the case of the mixed inoculation with *Methylobacterium* sp. OR01 and *M. extorquens* AM1 (Figure 4(b)). As revealed in the present study, *Methylobacterium* sp. OR01 is a pantothenate auxotroph and *M. extorquens* AM1 is prototrophic for not only pantothenate but also other B vitamins (Table 1). Under the tested conditions, the pantothenate auxotrophic *Methylobacterium* sp. OR01 dominated over the non-auxotrophic strain on plant leaves.

One reason why the auxotrophic strain was more effective at colonizing than the prototrophic strain might be due to that the auxotrophic strain does not need to consume energy for the synthesis of pantothenate or its precursors. Biosynthesis of one molecule of pantothenate requires 2 molecules of NADPH for generation of panthoate and one molecule of ATP for the condensation reaction between panthoate and β-alanine. Furthermore, the biosynthesis of one molecule of β-alanine from uracil requires one molecule of NADH. We also found that *Methylobacterium* sp. strain Rst is an auxotroph for biotin and pyridoxine (Table 1). Pyridoxine biosynthesis from pyridoxal requires 1 molecule of NADPH, and biotin biosynthesis from pimelic acid

requires 2 molecules of ATP.

Most strains of intestinal bacteria are known to be auxotrophs. For example, the lactic acid bacterium, *Leuconostoc citrovorum*, requires many amino acids, nucleobases, and vitamins for growth in synthetic medium [41]. Using co-culture experiments with the mutant and wild-type strains of *E. coli* and *Acinetobacter baylyi*, D'Souza *et al*. demonstrated that the loss of essential biosynthetic genes for a certain amino acids, nucleobases, or vitamins was generally beneficial when the required compounds were present in the growth environment [42]. In other words, the fitness advantage of the auxotrophic mutant increased more than that of the prototrophic wild type. As with the case of bacteria living in the intestinal nutrient environment, *Methylobacterium* sp. OR01, which is a dominant colonizer on plant leaves and can be directly transmitted from seeds to leaves [25], might have increased fitness by acquiring pantothenate and β-alanine, which are present in the phyllosphere, saving energy costs for biosynthesis of these compounds, and by possessing multiple pathways for β-alanine biosynthesis from several precursors available in the phyllosphere.

Author contributions

Y.Y. and H.I. performed the experiments. Y.Y., H.I., Y.S., and H.Y. designed the experiments and analyzed the data. Y.Y., Y.S., and H.Y. wrote the manuscript.

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514 **Figure legends**

515

516 **Figure 1.** β-Alanine auxotrophy of *Methylobacterium* sp. OR01.

517 (a) Biosynthesis of pantothenic acid. Pantothenic acid is synthesized by a
518 condensation reaction of pantoic acid and β-alanine. The *panC* encodes pantothenate
519 synthetase (EC: 6.3.2.1). (b) Growth of *Methylobacterium* sp. OR01.
520 *Methylobacterium* sp. OR01 was cultured in MM containing methanol as the sole
521 carbon source, supplemented with 15 μM each of pantothenate (open circles), β-alanine
522 (closed circles) or pantoate (open squares).

523

524 **Figure 2.** Pathways for bacterial β-alanine biosynthesis.

525 Red arrows indicate that homologous genes for enzymes involved in β-alanine
526 biosynthesis were found in the genome of *Methylobacterium* sp. OR01. Black arrows
527 indicate that the homologous gene was absent in the genome. Black frames indicate
528 compounds that could not restore the growth of *Methylobacterium* sp. OR01; Red
529 frames indicate compounds that could restore the growth of *Methylobacterium* sp.
530 OR01 (Figure 3).

531

532 **Figure 3.** Growth of *Methylobacterium* sp. OR01 supplemented with β-alanine
533 precursors.

534 *Methylobacterium* sp. OR01 was cultivated in MM containing methanol as a
535 carbon source and supplemented with 60 μM of each β-alanine precursor. The OD_{600}
536 values after cultivation for 72 h are shown.

537

538 **Figure 4.** Population of PPFMs on the *Arabidopsis* leaves.

539 (a) The sterilized seeds of *A. thaliana* were inoculated with *Methylobacterium* sp.
540 OR01 or *M. extorquens* AM1 and sowed onto Murashige and Skoog agar medium
541 without pantothenate. The plants were grown for 14 days and the fourth leaves were

542 removed from the plant and suspended in sterilized water. CFUs of the cell
543 suspension were measured as described in Materials and Methods. Box = 25th and
544 75th percentiles; bars = minimum or maximum values (if the minimum value is lower
545 than the 25th percentiles - 1.5 x box height or maximum value is higher than the 75th
546 percentile + 1.5 x box height, then bars = 25th percentile - 1.5 x box height and 75th
547 percentile + 1.5 x box height). Dot plot describes the CFUs/leaf for the individual
548 leaves. *Methylobacterium* sp. OR01, n=40; *M. extorquens* AM1, n=42. (b) The
549 sterilized seeds of *A. thaliana* were inoculated with a mixture of *Methylobacterium* sp.
550 OR01 (Km^r) and *M. extorquens* AM1 (Tet^r) [25], and sowed onto Murashige and Skoog
551 agar medium without pantothenate. The plants were grown for 14 days and the fourth
552 leaves were removed from the plant and suspended in sterilized water. CFUs of the
553 cell suspension were measured as described in Materials and Methods using MM agar
554 plates containing kanamycin (20 μg/mL) or tetracycline (10 μg/mL). Box = 25th and
555 75th percentiles; bars = minimum or maximum values (if the minimum value is lower
556 than the 25th percentiles - 1.5 x box height or maximum value is higher than the 75th
557 percentile + 1.5 x box height, then bars = 25th percentile - 1.5 x box height and 75th
558 percentile + 1.5 x box height). Dot plot describes the CFUs/leaf for the individual
559 leaves. *Methylobacterium* sp. OR01, n=50; *M. extorquens* AM1, n=50. n.d., not
560 detected.
561
562 **Figure 5.** Growth of *Methylobacterium* sp. OR01 in minimal medium supplemented
563 with various concentrations of pantothenate or β-alanine.
564 *Methylobacterium* sp. OR01 was cultivated in MM containing 0.5% methanol as
565 the carbon source supplemented with (a) pantothenate and (b) β-alanine. Each
566 concentration of pantothenate and β-alanine is indicated within the graphs.

Table 1. Pantothenate auxotrophy of *Methylobacterium* sp. strains isolated from living plant samples.

Strain	Source	Closely-related strain	16S rRNA identity (%)	Growth[a]		
				with VB mix[b]	without VB mix[c]	with pantothenate[d]
By	Yew leaf	*M. radiotolerans* strain 91a	99.7	+	-	+
B2B	Rice leaf	*M. radiotolerans* strain 91a	99.8	+	-	+
RS1	Rice seed	*M. fujisawaense* strain JoN18	100	+	-	+
C06	Rice leaf	*M. fujisawaense* strain JoN18	100	+	-	+
C16	Rice leaf	*M. fujisawaense* strain JoN18	100	+	-	+
C17	Rice leaf	*M. fujisawaense* strain JoN18	99.9	+	-	+
In1	Yew leaf	*M. radiotolerans* strain 91a	100	+	-	+
OR01	Red perilla seeds	*M. fujisawaense* strain JoN18	100	+	-	+
Sh	Camellia leaf	*M. radiotolerans* strain 91a	99.9	+	-	+
C1	Clover leaf	*M. extorquens* AM1	99.9	+	+	+
Dw	Duck weed	*M. adhaesivum* strain 45i	100	+	+	+
Rfw	Paddy water	*M. populi* strain BJ001	99.7	+	+	+
Rst	Rice stem	*M. platani* strain JoF11	99.8	+	-	-
AM1	-	*M. extorquens* AM1	100	+	+	+

[a] +, growth; -, no growth.

[b] Each strain was cultured in MM with VB mix. VB mix contains thiamine (B_1), niacin (B_3), Ca-pantothenate (B_5), pyridoxine HCl (B_6), biotin (B_7), cobalamin (B_{12}), inositol (B-h), and *p*-amino benzoate (B-x). Their concentrations are described in Materials and Methods.

[c] Each strain was cultured in MM without VB mix.

[d] Each strain was cultured in MM with pantothenate.

Table 2. Concentration of pantothenate and its precursors in the suspension of the leaves of *A. thaliana.*

Compounds	Concentration[a] (pmol/g fresh leaves)
pantothenate	$(1.5 \pm 0.5) \times 10^{1}$
β-alanine	$(1.1 \pm 0.7) \times 10^{3}$
spermine	n.d.[b]
spermidine	$(3.9 \pm 1.4) \times 10^{1}$
5,6-dihydrouracil	n.d.
N-carbamoyl-β-alanine	$(7.8 \pm 4.3) \times 10^{0}$
3-hydroxypropanoate	$(2.7 \pm 0.4) \times 10^{1}$

[a] The mean ± standard deviation of the mean (β-alanine: n=3, other compounds: n=5).

[b] Not detected.

Fig. 1

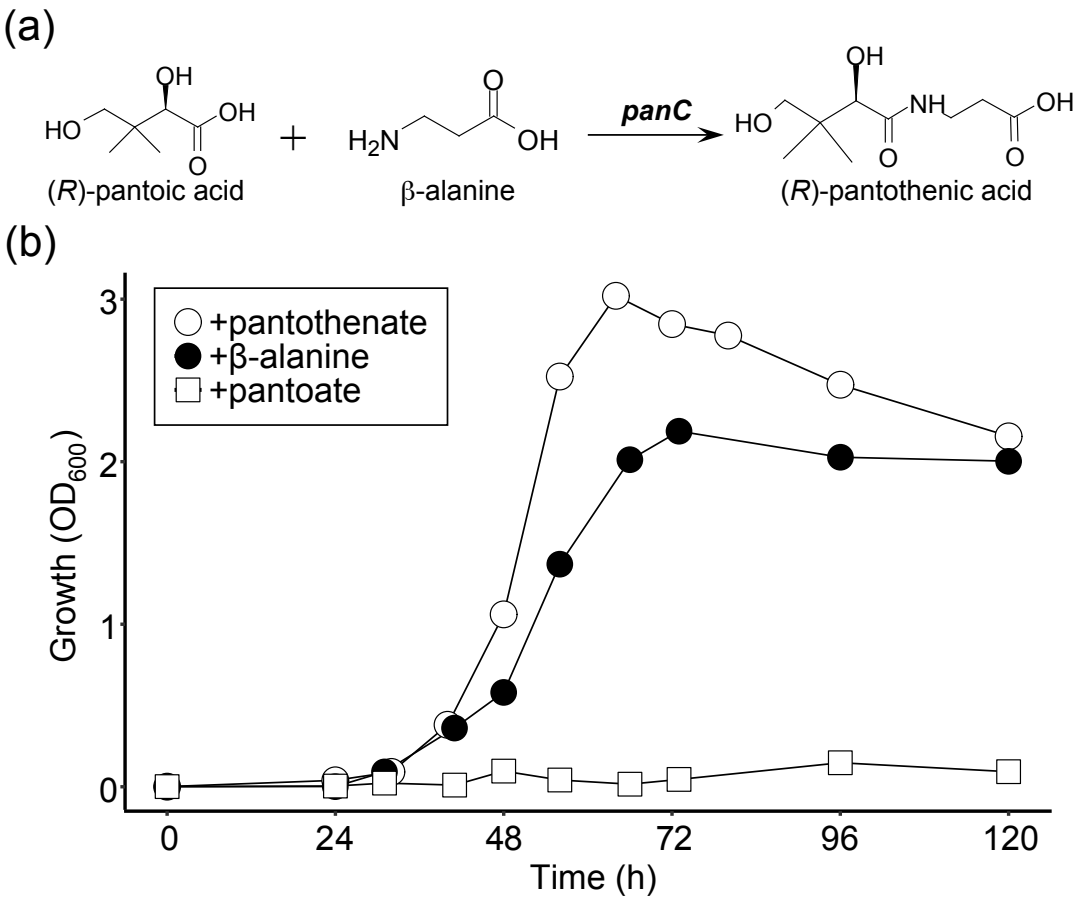

Fig. 2

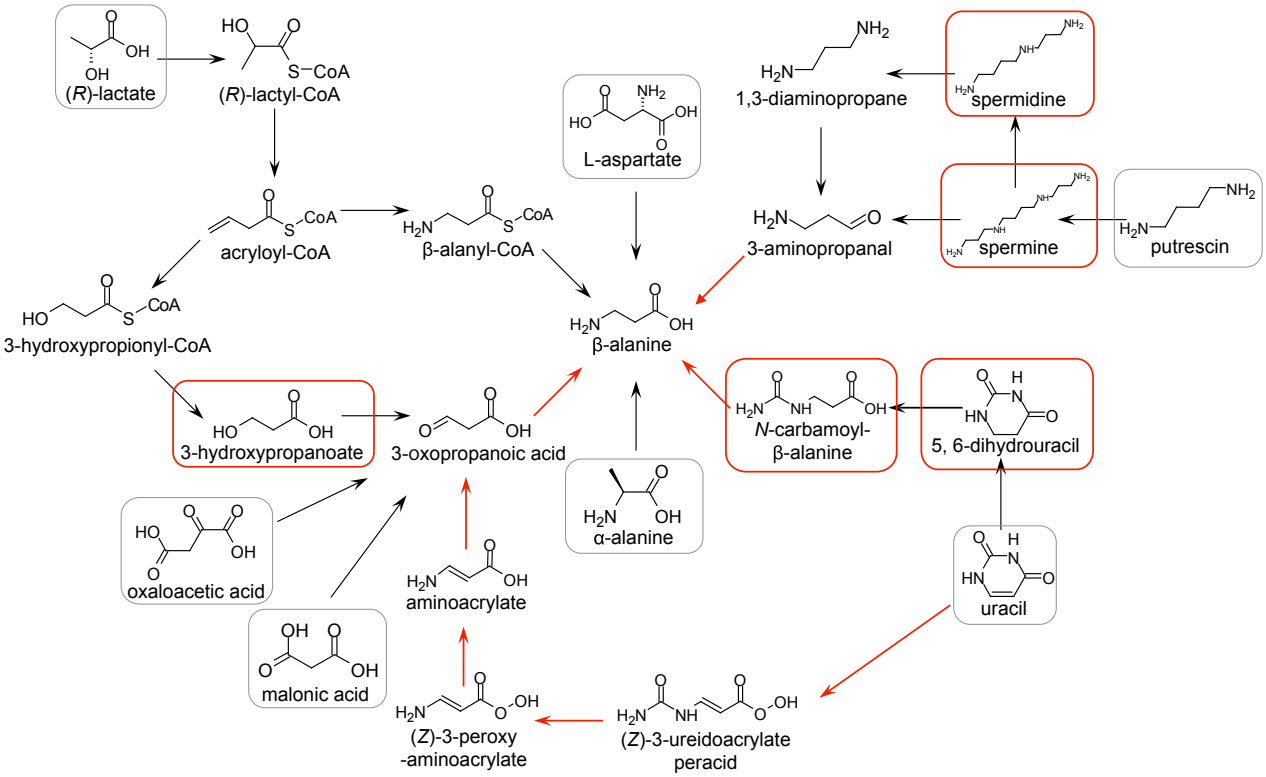

Fig. 3

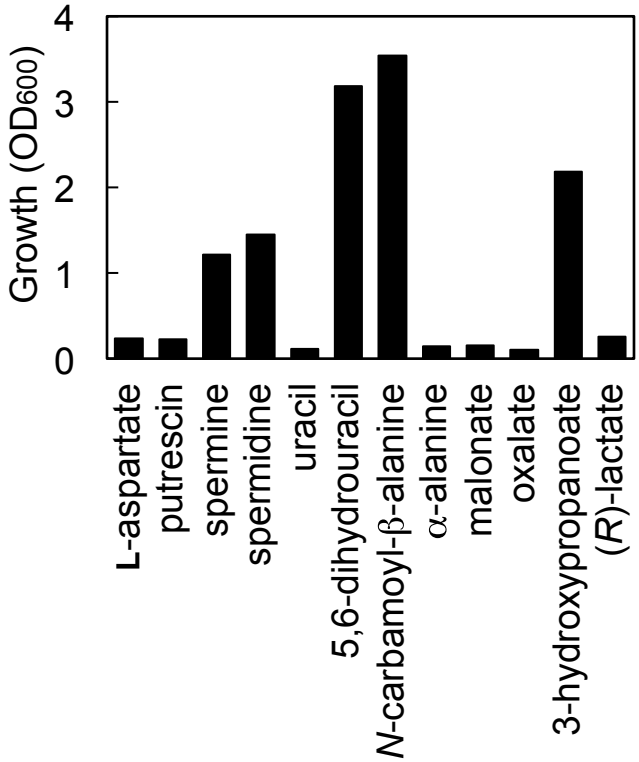

Fig. 4

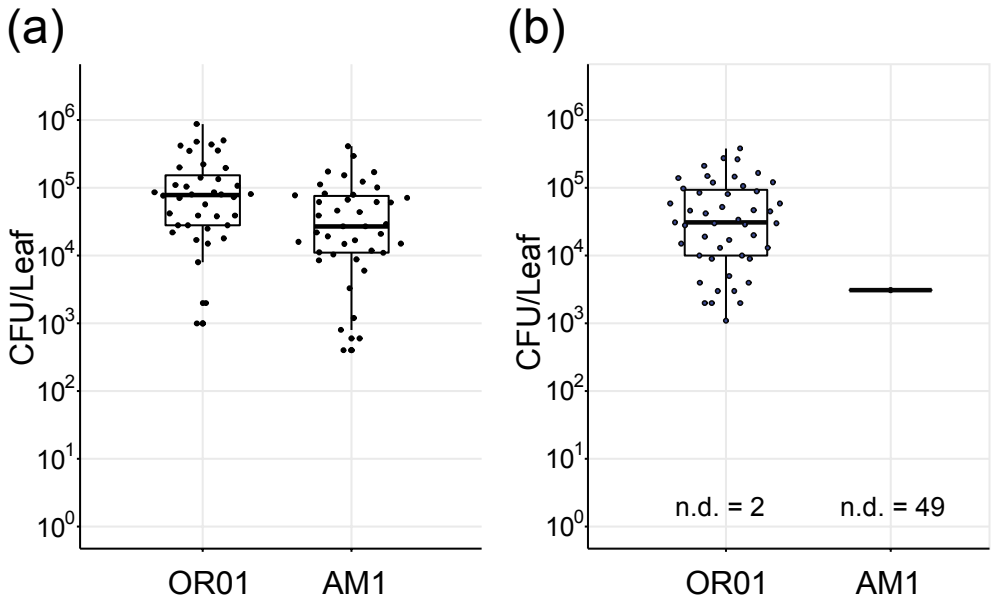

Fig. 5

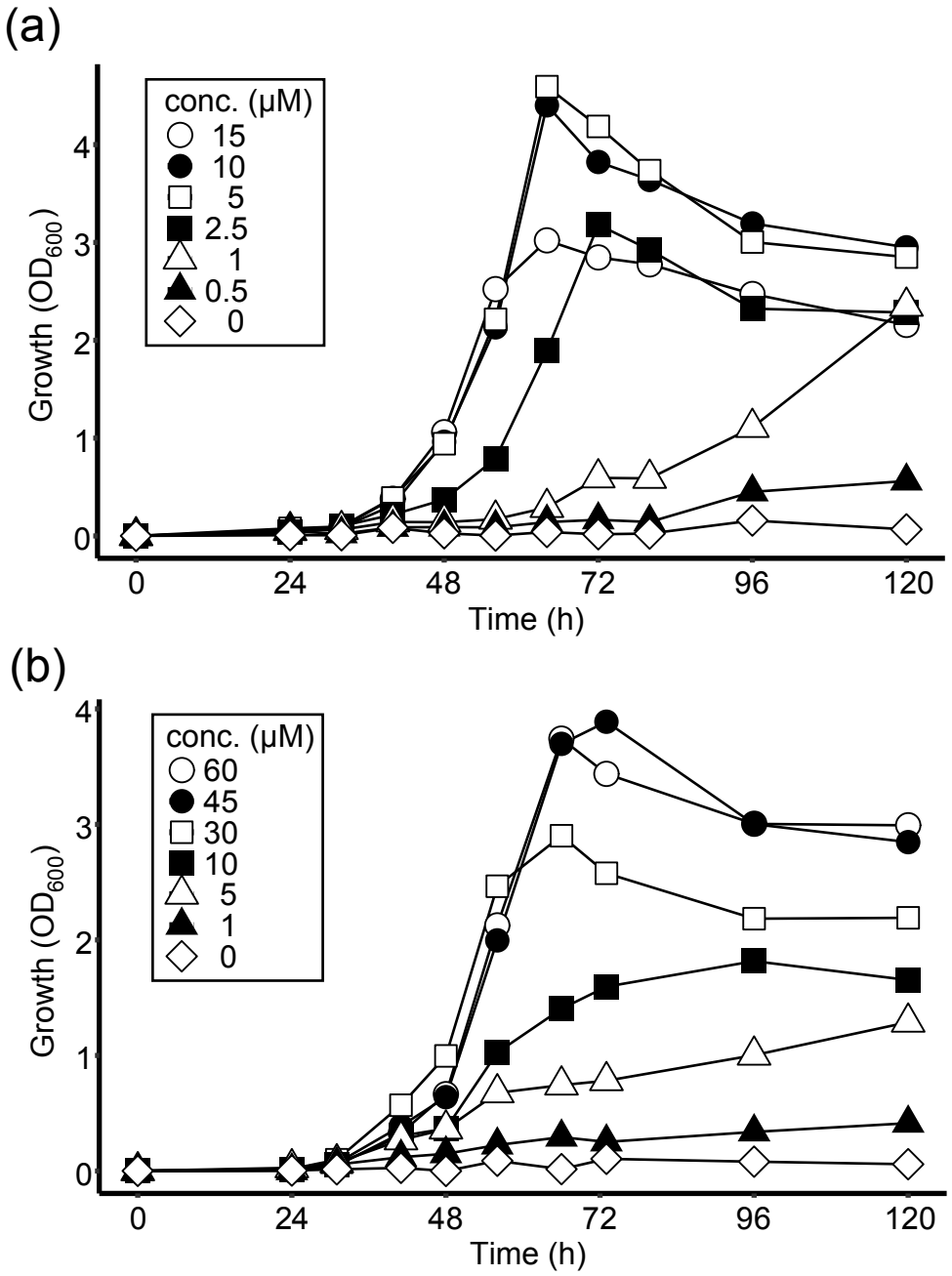

Table S1. Parameters for LC-MS/MS analysis.

Compounds	Q1 (m/z)	Q3 (m/z)	DP (V)	CE (V)	CXP (V)	GS1	GS2	CUR	TEM (°C)	IS (V)
pantothenate	217.844	87.900								
N-carbamoyl-β-alanine	130.900	88.100	-40	-15	-9	30	80	40	400	-4500
3-hydroxypropanoate	88.900	58.900								
spermine	203.200	129.100								
spermidine	146.163	71.900	26	17	6	30	80	40	500	4500
5,6-dihydrouracil	115.200	69.900								
β-alanine (derivatized)	210.000	121.000	60	29	10	70	70	10	600	5500

Q1, mass of precursor ion: Q3, mass of product ion: DP, declustering potential: CE, collision energy: CXP, collision exit potential: GS1, ion source gas 1 (nebulizer gas): GS2, ion source gas 2 (heater gas): CUR, curtain gas: TEM, temperature: IS, ion spray voltage.